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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/02, 1/68, G01N 33/577	A1	(11) International Publication Number: WO 95/14104 (43) International Publication Date: 26 May 1995 (26.05.95)
(21) International Application Number: PCT/US94/13215 (22) International Filing Date: 16 November 1994 (16.11.94) (30) Priority Data: 08/153,183 16 November 1993 (16.11.93) US (60) Parent Application or Grant (63) Related by Continuation US 08/153,183 (CIP) Filed on 16 November 1993 (16.11.93) (71) Applicant (for all designated States except US): CHILDREN'S MEDICAL CENTER CORPORATION [US/US]; 300 Longwood Avenue, Boston, MA 02115 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): HARRIS, H., William [US/US]; 87 Haven Street, Dover, MA 02030 (US). PARE- DES, Ana [US/US]; 14271 S.W. 36th Street, Miami, FL 33175 (US). PIQUERAS, Anabel [ES/US]; 801 South Bayshore Drive, Miami, FL 33131 (US). OZKAYNAK, En- gin [US/US]; 44 Purdue Drive, Milford, MA 01757 (US). SOMERS, Michael [US/US]; 64 Greenhill Road, Westwood, MA 02090 (US).	(74) Agents: BROOK, David, E. et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US). (81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ). Published <i>With international search report.</i> <i>Before the expiration of the time limits for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>	
(54) Title: METHOD OF IDENTIFYING A SUBSTANCE CAPABLE OF INDUCING BONE FORMATION (57) Abstract <p>A rapid and sensitive method for screening candidate substances to determine their effect on OP-1 mRNA expression, or OP-1 protein synthesis, and thus, determining the effect of the substance on bone formation and methods of therapeutic treatment for individuals suffering from bone disease.</p>		

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METHOD OF IDENTIFYING A SUBSTANCE CAPABLE OF
INDUCING BONE FORMATION

Background of the Invention

Osteogenic protein-1 (OP-1) is a member of the transforming growth factor (TGF)- β family which induces de novo bone formation by stimulating collagen synthesis and proliferation and differentiation of osteoblasts. (Sampath, T.K., et al., J. Biol. Chem., 267:20352-20362 (1992)). The OP-1 protein was initially isolated in demineralized bone matrix. (Sampath, T.K., J. Biol. Chem., 265:13198-13205 (1990)). The murine OP-1 gene encoding the homolog of human OP-1 (Özkaynak, E., et al., EMBO J. 9:2085-2093 (1990)) was isolated from cDNA and genomic libraries using human OP-1 cDNA as a probe. Northern blot analysis with murine OP-1 cDNA showed two major species of 2.4 kb and 4.0 kb, which may represent alternative splices. Tissue specific expression studies revealed maximal levels of OP-1 in the kidney (Özkaynak, E., et al., Biochem. Biophys. Res. Comm., 30:116-123 (1991)), a unique feature among the so called bone morphogenetic proteins (BMPs) (Özkaynak, E., et al., J. Biol. Chem., 267:25220-25227 (1992)).

The expression of OP-1 in the kidney suggests its potential role as a link between this organ and cellular elements in the skeleton, and may explain an earlier observation (Huggins, Arch. Surg., 22:377-408 (1931)) regarding the capability of the urinary tract epithelium to induce new bone formation.

However, the specific localization of OP-1 mRNA and protein species along the nephron, as well as the role of OP-1 in the cell, and molecular biology of the kidney remain unknown.

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Moreover, the study of OP-1 expression in the kidney has been limited by the unavailability of an acceptable model system. To understand the function of different nephron segments, it is necessary to isolate and culture
5 cells representative of the many different cell types that comprise the nephron. A major limitation in this regard is that cells isolated from microdissected kidney tubules have a limited life span in culture. Furthermore, expression studies of osteogenic proteins have been
10 difficult to reproduce in primary cell cultures due to minimal levels of OP-1 mRNA expression under physiological conditions. In vitro transfection of cells with agents such as viruses or oncogenes produce immortalized cell lines, but often these cells lose their differentiated
15 functional characteristics. (Gullans, S.R., et al., Am. J. Physiol., 265 (1993)). Thus, the need exists to determine the specific nephron location and cell type responsible for OP-1 mRNA expression, and to devise a model assay system for the rapid screening and
20 identification of substances which induce new bone formation by regulating OP-1 mRNA expression and OP-1 protein synthesis.

Summary of the Invention

The present invention relates to Applicants'
25 characterization of mRNA expression and protein synthesis of osteogenic protein-1 (OP-1) in kidney sections. Additionally, by screening a variety of cultured renal epithelial cell lines, Applicants have found a cell line with markedly high baseline OP-1 mRNA expression. As a
30 result of Applicants' work, presented herein, it has been determined, for the first time, that OP-1 mRNA transcripts and proteins are present in the renal medulla and that inner medullary collecting duct (IMCD) cells synthesize abundant OP-1 protein.

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As a further result of Applicants' work, a model assay system is now available to rapidly identify substances capable of inducing new bone formation by the evaluation of the effect of test substances on the
5 expression of OP-1 mRNA expression and OP-1 protein synthesis in inner medullary collecting duct cells.

In one embodiment of the present invention, identification of a substance capable of inducing new bone formation is determined by evaluating the effect the
10 substance has on inducing OP-1 mRNA expression in mammalian cells. Mammalian cells are grown in culture and the substance to be tested (i.e., the test substance) is introduced into the cell culture medium, or into the cell. The test substance is introduced in an amount which would
15 produce a detectable increase in the expression of OP-1 mRNA if the substance possesses OP-1 mRNA inducing properties. The cells are maintained in culture under conditions which support cell growth and RNA transcription, for a period of time sufficient for RNA
20 transcription to occur. The cellular RNA is then isolated, and the amount of OP-1 mRNA expressed by the cultured cells is determined. The amount of OP-1 mRNA expressed by the cultured cells in the presence of test substance is compared to the amount of OP-1 mRNA expressed
25 by cultured cells under similar conditions, but without the presence of the test substance, to determine the effectiveness of the test substance to induce OP-1 mRNA.

In another embodiment, identification of a substance capable of inducing new bone formation is determined by
30 evaluating the effect the substance has on inducing the synthesis OP-1 protein in mammalian cells. Mammalian cells are grown in culture and the test substance is introduced into the cell culture medium, or into the cell. The test substance is introduced in an amount which would
35 produce a significant increase in the synthesis of OP-1

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protein if the substance possesses OP-1 protein inducing properties. The cells are maintained in culture under conditions which support cell growth and protein synthesis and the amount of OP-1 protein synthesized by the cultured
5 cells is determined. The amount of OP-1 protein synthesized by the cultured cells in the presence of test substance is compared to the amount of OP-1 protein synthesized by cultured cells under similar conditions, but without the presence of the test substance, to
10 determine the effectiveness of the test substance to induce OP-1 protein synthesis.

In another embodiment, the present invention provides a method of therapeutically treating a patient with bone disease, such as osteoporosis, to induce bone growth (bone
15 morphogenesis). This method comprises administering to the patient an effective amount of a substance capable of inducing bone growth by stimulating OP-1 mRNA expression, or OP-1 protein synthesis. Conversely, in another embodiment, the present invention provides a method of
20 therapeutically treating a patient with a bone disease, characterized by overproduction of bone (i.e., osteopetrosis) to suppress bone formation. This method comprises administering to the patient an effective amount of a substance capable of suppressing bone growth by
25 suppressing OP-1 mRNA expression, or OP-1 protein synthesis.

Thus, as described herein, the present invention provides a rapid and sensitive method for screening candidate substances (test substances) to determine their
30 effect on OP-1 mRNA expression, or OP-1 protein synthesis and secretion, and thus, determine the effect of the substance on bone formation. It also provides a model system to study the interaction of OP-1 protein with other known regulators of bone homeostasis, such as vitamin D,
35 calcitonin, parathyroid hormone or growth factors such as

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TGF- β , and other substances like mineralocorticoids, and the response of OP-1 production to changes in pH, calcium, phosphorous concentration. Finally, the present invention provides a therapeutic treatment for patients suffering from bone disease.

Brief Description of the Figures

Figure 1A and 1B depicts the murine OP-1 cDNA sequence (SEQ ID NO: 1) and encoded amino acid sequence (SEQ ID NO: 2).

10 Figure 2 is a photograph of a Northern blot showing the results of Poly A⁺ RNA from kidney (cortex, medulla and papilla), lung, liver, brain, ovary and small intestine probed with OP-1 cDNA.

Figure 3A and 3B depicts two graphs showing the ratio
15 OP-1/ β -actin signal calculated for the 2.4 and the 4.0 kb OP-1 transcripts for each tissue (n=6).

Figure 4 is a photograph of an immunoblot showing the results of Western blot analysis showing that 12G3 recognizes OP-1 protein. The arrows denote the positions
20 of molecular weight standards expressed as molecular mass X 10³.

Figure 5 is a photograph of an immunoblot showing that mouse monoclonal antibody 12G3 identifies multiple OP-1 protein bands in homogenates from rat kidney and
25 cultured mouse inner medullary collecting duct (IMCD) cells. The small arrowheads point to the top (T) and bottom (B) of the gel.

Figure 6A-6D is a series of micrographs showing that mouse monoclonal 12G3 localizes OP-1 staining to cells in
30 kidney glomeruli, tubules and vessels in rat kidney.

Figure 7A-H is a series of micrographs showing localization of OP-1 expression in mouse kidney by *in situ* hybridization.

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Figures 8A-B are photographs of a Northern blot showing the results of northern analysis of MDCK and IMCD cells probed with OP-1 cDNA.

Detailed Description of the Invention

5 Applicants have determined, by expression studies in dissected kidney sections and immunohistochemistry analysis, that the renal medulla contains high levels of the OP-1 protein. Specifically, Applicants have demonstrated that OP-1 mRNA is highly expressed in inner
10 medullary collecting duct cells. Based on Applicants results, a rapid and sensitive method for screening candidate substances to determine their effect on OP-1 mRNA expression, or OP-1 protein synthesis, and thus, determining the effect of the substance on bone formation
15 in now provided.

As described in detail in Example 1, Poly A⁺ RNA extracted from kidney sections (cortex, medulla and papilla) were analyzed by Northern blot analysis using a ³²P-labeled OP-1 cDNA probe (SEQ ID NO: 1). The probe,
20 shown in Figure 1, b.p. 370-1050, is a 680 b.p. fragment that covers two thirds of the pro-domain and one third of the mature murine OP-1 cDNA, immediately upstream to the highly conserved 7-cysteine domain present in all members of the TGF- β superfamily (Özkaynak, E., et al., Biochem.
25 Biophys. Res. Commun., 30:116-123 (1991) the teachings of which are incorporated herein by reference). To compare kidney expression of OP-1 mRNA with extrarenal expression, lung, liver, brain, ovary and small intestine mRNA were also isolated and subjected to hybridization with the OP-1
30 cDNA probe. After hybridization with the OP-1 cDNA probe, the same membranes were stripped and reprobed with mouse β -actin (Figure 2, lower row) to control for mRNA content and quality. As illustrated in Figure 2, Northern blot analysis identified the OP-1 transcript (2.4 and 4.0 kb)

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in all tissues of the normal rats surveyed. However, the signal was markedly higher in kidney sections, and OP-1 mRNA expression was greatest in the renal medulla, followed by cortex and papilla.

5 Figure 3A and 3B shows the percentage of OP-1 mRNA expression (mean \pm SEM, n=6 animals) for the rat tissues studied. The highest value was taken as 100%, and the percentages for the other tissues were derived by relating their ratios to the highest one. The calculated means \pm 10 SEM of the mRNA percentages in the tissues analyzed is shown in Figure 3. OP-1 mRNA expression for the 2.4 and 4.0 kb transcripts, respectively, was highest in the renal medulla followed by cortex and papilla. The relative mRNA expression for the 2.4 and 4.0 kb transcripts was 15 respectively higher in the renal medulla ($77.6 \pm 11.2\%$ and $65.0 \pm 8.3\%$) followed by cortex ($72.7 \pm 12.2\%$ and $41.6 \pm 7.8\%$) and papilla ($62.7 \pm 8.3\%$ and $53.9 \pm 11.9\%$). Relative mRNA expression in kidney sections was significantly greater than that of extrarenal tissues 20 ($p=0.0002$ for the 2.4 kb band and $p=0.0001$ for the 4.0 kb band). Brain showed the lowest signal (9.0 ± 3.3 and 8.3 ± 3.0) whereas ovary (18.8 ± 5.2 and 15.1 ± 3.1) elicited the highest extrarenal expression. Results for liver (15.2 ± 5.2 and 8.2 ± 2.0), intestine (12.4 ± 3.7 and 8.1 ± 2.3) 25 and lung (10.8 ± 3.0 and 8.2 ± 2.0) were more variable.

Although the putative amino acid sequence of the OP-1 cDNA has been deduced and OP-1 expressed in transfected mammalian cells in culture (Özkaynak, E., et al., EMBO J., 9:2085-2093 (1990); Sampath, T.K., et al., J. Biol. Chem., 30 267:20352-20362 (1992); Jones, W.K., et al., Bone and Mineral Research, S348. (1993)), little is known with regard to endogenous processing of OP-1 by various tissues. The OP-1 cDNA predicts a primary translation polypeptide of 49 kD that includes a signal secretory 35 sequence (29 A.A or ~ 3.5 kD), a pro domain (~ 270 A.A. or

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31 kD) containing two polybasic proteolytic processing sites and a TGF- β domain (138 A.A. or 16 kD). OP-1 is a glycoprotein (Sampath, T.K., et al., J. Biol. Chem., 265:13198-13205 (1990); Sampath, T.K., et al., J. Biol. Chem., 267:20352-20362 (1992)) possessing 4 distinct potential glycosylation sites that would increase the apparent molecular mass of any of these OP-1 polypeptides.

The processing of human OP-1 has been partially characterized after its expression in either cultured CHO or BCC-1 cells (Sampath, T.K., et al., J. Biol. Chem., 267:20352-20362 (1992); Jones, W.K., et al., Bone and Mineral Research, S348. (1993)). These data suggest the major intracellular form of OP-1 in CHO cells is polypeptide of approximately 50 kD that is dimmerized upon its secretion. This OP-1 complex undergoes further cleavage and processing in an acidic intracellular vesicular compartment and is finally secreted as a complex of mature OP-1 homodimers.

A monoclonal antibody, antibody 12G3, was produced as described in detail in Example 2. To verify the specificity of mouse monoclonal antibody 12G3 for OP-1 proteins, 0.5 ugs of spent media derived from CHO cells expressing recombinant human OP-1 was probed. (Sampath, T.K., et al., J. Biol. Chem., 267:20352-20362 (1992); Jones, W.K., et al., Bone and Mineral Research, S348. (1993)) As shown in Figure 4, monoclonal 12G3 recognized 3 bands of 65 kD, 44 kD and broad band of 27-19 kD. As reported previously (Jones, W.K., et al., Bone and Mineral Research, S348. (1993)). These bands correspond to the glycosylated primary translation OP-1 polypeptide (65 kD), pro-OP-1 (44 kD) and mature OP-1 (27-19 kD) proteins. These data demonstrate that 12G3 recognizes an epitope present on both primary and mature OP-1 proteins.

To obtain evidence for the presence of OP-1 species, as well as mRNA, in the kidney, homogenates of kidney

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tissue were probed with monoclonal 12G3. As shown in Figure 5, whole kidney (lane 1) as well as renal cortex (lane 2), medulla (lane 3) and papilla (lane 4) with monoclonal antibody 12G3 specifically reacted with 12G3.

5 Either omission of 12G3, or substitution of another irrelevant monoclonal resulted in the complete absence of bands. Monoclonal 12G3 recognized 6-7 major OP-1 bands including species of 53 kD, 45 kD, 36 kD, 34 kD, 2 closely spaced bands of 19-17 kD and a 15 kD band of variable

10 intensity. The overall pattern of OP-1 bands was similar in homogenates of renal cortex and medulla (lanes 2 and 3, of Figure 5). In contrast, the 53 kD was less intense in the renal papilla (lane 4, of Figure 5) as compared to renal cortical and medullary homogenates.

15 Several of the bands identified in kidney homogenates by 12G3 possess molecular masses identical to that described for OP-1 species harvested from OP-1 producing CHO cells shown in Figure 4. The major OP-1 bands of 45 kD, 19 kD and 17 kD are identical to those displayed in

20 Figure 3 and likely correspond to pro-OP-1 protein as well as the 2 mature glycosylated OP-1 products. Previous data (Sampath, T.K., et al., J. Biol. Chem., 267:20352-20362 (1992); Jones, W.K., et al., Bone and Mineral Research, S348. (1993)) have identified a 15 kD OP-1 band as

25 possessing multiple amino termini and thus is likely the result of additional proteolytic degradation. The 53 kD band is similar to the molecular mass of the primary translation OP-1 polypeptide and less intense bands in the 65 kD region of Figure 4 may correspond to glycosylated

30 forms of this initial OP-1 polypeptide shown in Figure 4.

The bands of 36 and 34 kD identified by 12G3 have not been reported as major bands in immunoblotting analyses of CHO cell homogenates containing processed OP-1 polypeptides (Jones, W.K., et al., Bone and Mineral

35 Research, S348. (1993)). Several possibilities could

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account for the apparent molecular masses on SDS-PAGE. These include either differences in the carbohydrate content between endogenous and CHO cells OP-1 proteins, incomplete reduction of 17 kD and 19 kD dimers or
5 additional OP-1 processing steps that are not present in CHO cells. For example, at least 2 polybasic proteolytic cleavage sites are present in the prodomain of OP-1 (Özkaynak, E., et al., Biochem. and Biophysical Research Comm., 179:116-123 (1991)) that may be utilized by kidney
10 cells expressing OP-1.

As shown in Figure 6A-D, immunohistochemistry staining using monoclonal 12G3 localizes OP-1 to glomeruli and adjacent large vessels (Panel B) as well as epithelial cells of renal tubules (Panel C) and small vessels located
15 in medullary portions of the kidney (Panel D). Glomerular OP-1 staining appears to be localized in both epithelial cells in Bowman's capsule as well as other cells within the glomerular tuft. In addition, endothelial and smooth muscle cells of vessels including the arterioles of the
20 juxtaglomerular apparatus display prominent OP-1 staining.

Mouse monoclonal 12G3 is unsuitable for use as a probe for mouse tissues. However, *in situ* hybridization analysis of serial sections of mouse kidney was performed to determine whether OP-1 mRNA expression displayed a
25 pattern similar to that observed for OP-1 protein in rat kidney. As shown in Figure 7A-H, paraffin sections of whole kidney were hybridized with either antisense (Panels B and F) or sense (Panels D and H) RNA synthesized from the same cDNA probe used in Figure 2. In a fashion
30 similar to that demonstrated by northern analysis of rat kidney shown in Figure 2, specific hybridization by the OP-1 antisense probe was observed in the renal cortex, medulla and papilla. Specific labeling of tubular and/or vascular elements in the papilla and inner medulla of the
35 kidney were readily discernable as shown in Panel F of

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Figure 7. There was also specific labeling of both medullary and cortical structures including areas corresponding to glomeruli as shown by the large open arrows in Panels A and B. Specific OP-1 hybridization also appeared to be distributed in a nonhomogeneous manner that was especially prominent in the inner medulla and papilla (see Panel F). This pattern of OP-1 mRNA expression is similar to that observed in rat kidney using immunohistochemistry as shown in Figure 6.

10 To obtain a kidney derived cell line to further study OP-1 expression, confluent cells from 3 types of cultured epithelial cells were screened by northern and immunoblot analyses using the cDNA and 12G3 antibody OP-1 probes described above. Opossum kidney (OK) cells, canine (MDCK

15 cells and an SV-40 transformed mouse IMCD cell line each form polarized epithelia. Both OP and MDCK cells have been utilized extensively in studies of epithelial cell polarity and transport. The IMCD cell line is derived from the cells of the IMCD and it retains many

20 characteristics of this nephron segment including increased transepithelial resistance in response to dexamethasone, inhibition of sodium flux by amiloride and atrial natriuretic peptide and the ability to adapt to hypertonicity (Rauchman, M.I., Am. J. Physiol., 265-271

25 (1993)).

In contrast to OK cells where no OP-1 expression was detected, both MDCK and IMCD cell poly A⁺ RNA contained both 4.0 and 2.4 kb OP-1 transcripts as shown in Figure 8. OP-1 expression by MDCK cells confirms previous reports

30 using a canine OP-1 cDNA probe (Ishibashi, K., et al., Biochem. and Biophysical Research Comm., 193:235-239 (1993)). However, OP-1 expression by IMCD cells (Right Panel) was consistently larger when compared to MDCK cells (Left Panel) even under conditions of reduced stringency

35 to account for species differences.

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Immunoblot analysis using 12G3 also detected the presence of 4 OP-1 species in IMCD cell homogenates (lane 5, Figure 5) that were identical to the pattern exhibited by rat kidney homogenates (lanes 1-4, Figure 5). The 5 major IMCD OP-1 bands included bands of 36 kD, 19 kD, 17 kD and 15 kD. Although IMCD cells did display 12G3 reactive bands of 53 kD and 45 kD, both were less intense as compared to cortical (lane 2) and medullary (lane 3) homogenates. The abundance of OP-1 protein species in 10 IMCD cells is reflected in the fact that lane 5 contains approximately one half the total protein content of lanes 1-4 of Figure 5.

The observation that most of the antibody localized to tubular structures in medullary rays, plus the high 15 baseline mRNA expression found in IMCD cells, indicates that tubular epithelial cells in the renal medulla are an important source of the OP-1 protein. Thus, Applicants have determined that renal epithelial cells, in particular, tubular epithelial cells in renal medulla 20 express high levels of OP-1 mRNA and OP-1 protein. As a result of this work,, a method is now available to identify substances capable of inducing bone formation by determining the effect a substance has on OP-1 mRNA expression, or OP-1 protein synthesis.

25 As used herein, the term "effect on OP-1 mRNA expression" means effect on the transcription of genetic information from DNA to mRNA. As used herein, the term "effect on OP-1 protein synthesis" means the effect on the translation of mRNA to protein molecules, as well as 30 effects on the biological activity of the protein molecule, or secretion of protein molecules from the cell.

OP-1 protein is a potent bone morphogen member of the transforming growth factor beta (TGF- β) superfamily and induces de novo bone formation by stimulating collagen 35 synthesis and the proliferation and differentiation of

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osteoblasts. Thus, upregulation of the expression of OP-1 mRNA, or an increase of OP-1 protein synthesis, provides a means to induce new bone formation. Alternatively, downregulation of OP-1 mRNA expression, or a decrease in
5 OP-1 protein synthesis, provides a means to suppress new bone formation. Thus, as used herein, substances that affect OP-1 mRNA expression, or OP-1 protein synthesis also affect bone formation.

The regulation of expression of OP-1 mRNA can be
10 accomplished by any method that regulates the transcription of OP-1 DNA to mRNA. Upregulation of the expression of OP-1 mRNA can be accomplished, for example, by a transcriptional activator factor, such as an ancillary protein necessary to initiate the
15 transcriptional process. Upregulation can also be accomplished, for example, by removal of a transcriptional factor which prevents initiation of the transcriptional process. For example, the upregulation of OP-1 mRNA expression can be accomplished by introducing into the
20 cell an antisense oligonucleotide which binds to a gene encoding a regulatory protein, that, when present, binds to OP-1 DNA and prevents initiation of transcription of OP-1 DNA to OP-1 mRNA. Removal of this regulatory protein thus upregulates the expression of OP-1 mRNA expression.
25 Alternatively, removal of the regulatory protein can be accomplished by introducing into the cell and an antibody, or antibodies, that bind to the regulatory protein and, thus, block, or neutralize, its activity. Removal of this regulatory protein initiates transcription of OP-1 DNA to
30 mRNA, and, thus, induces new bone formation.

The expression of OP-1 mRNA can also be downregulated by any method that which regulates the transcription of OP-1 DNA to mRNA. Downregulation of mRNA expression can be accomplished, for example, by sense, or antisense, DNA
35 complementary to a portion of OP-1 DNA, which hybridizes

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with all, or a portion, of the OP-1 gene, thus, preventing transcription.

The regulation of synthesis of OP-1 protein can be accomplished by any method that regulates protein synthesis, or regulates the biological activity of the protein, or the secretion of protein from a cell. For example, the synthesis of OP-1 protein can be regulated by substances that affect the translation of mRNA to protein, such as the binding of an antisense oligonucleotide to OP-1 mRNA, thus preventing translation. Alternatively, the biological activity of OP-1 protein can be altered by a substance that interferes with the maturation (e.g., biological activity) of the protein, such as cleavage of the pro region, or glycosylation of the protein. Moreover, regulation can occur by substances that stimulate, or inhibit secretion of OP-1 protein from the cell.

The method for the identification of substances capable of inducing new bone formation is performed as follows.

Renal medullary cells are grown under standard laboratory conditions at pH 7.4, at 37°C in 5% CO₂, in Dulbecco's Modified Eagle's Medium (DMEM) as described in Example 1. Renal medullary cells can be obtained from a variety of sources. These include, for example, dissecting mammalian kidneys and establishing primary renal medullary cell cultures, or using a cell line such as the Madin-Darby canine kidney (MDCK) cell line. Applicants have demonstrated that inner medullary collecting duct (IMDC) cells exhibit significantly higher expression levels of OP-1 mRNA and OP-1 protein than other kidney cells. A convenient source of IMDC cells, suitable for use in the present invention, is the mIMCD-3 cell line derived from a transgenic SV-40 mouse as described in Gullans, S.R., et al., Am. J. Physiol., 265 (1993), the

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teachings of which are incorporated herein by reference. Alternatively, other culture media can be used to practice this assay. Selection of suitable culture medium will depend on the ability of the medium to support growth of 5 renal cells and to provide an environment which permits the test substance to detectably affect OP-1 mRNA expression or OP-1 protein synthesis, and thus, induce bone formation.

Substances identified by the method described herein, 10 capable of inducing new bone growth formation, are substances which possess properties which affect the expression of OP-1 mRNA or synthesis of OP-1 protein. Candidate substances (test substances) can be oligonucleotides, peptides, proteins, small molecules or 15 organic molecules, including molecules that alter the pH, or ionic strength, of the cellular environment. For example, a test substance can be a peptide hormone (e.g., parathyroid hormone, anti-diuretic hormone, or atrial natriuretic peptide) or a steroid hormone such as vitamin D, 20 or one of its active metabolites. Mineralocorticoids can also be tested in IMCD cells. Minerals such as calcium and phosphorous, as well as substances that affect calcium and phosphorous concentration in the cellular environment, such as chelating agents, can also be tested. Test 25 substances can also be antisense oligonucleotides or antibodies. Accordingly, a test substance can be any substance reasonably believed to affect bone formation. However, the candidate substance must be sufficiently soluble in an aqueous medium to allow a sufficient amount 30 to dissolve so that the effect of the substance on OP-1 mRNA expression, or OP-1 protein synthesis, can be determined.

The test substance is then introduced into the culture medium, in sufficient amount and in a form that 35 allows the test substance to affect the expression of OP-1

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mRNA or synthesis of OP-1 protein. A sufficient amount of the test substance, (e.g., an effective amount) is determined for each individual substance to be tested. For example, it is known that 10^{-7} M atrial natriuretic peptide (ANP) inhibits luminal-to-basal transport of Na in IMCD cells. (Gullans, S.R., Am. J. Physiol., 265 (1993). Thus, 10^{-7} M ANP would be a reasonable amount of ANP to be tested in the method of the present invention. This substance would also be tested in a greater or lesser amount than 10^{-7} M to determine the optimum dose of ANP effective to induce bone formation.

The test substance must be in a form so as to allow the substance to affect the OP-1 mRNA expression or OP-1 protein synthesis. Preferably, the test substance is sufficiently soluble to allow an effective amount of substance to dissolve in the aqueous culture medium and subsequently to enter into the IMCD cell. However, the test substance can be introduced into the culture medium, or the cell via other routes, such as encapsulation by a liposome, or by other standard methods used to introduce substances into culture media and cultured cells.

Once the test substance is introduced into the culture medium or cell, the test culture is incubated under conditions (e.g., time, temperature, CO₂ content) suitable for growth of the cells. Incubation conditions and duration will depend on the nature of the individual substance being tested. A control culture is run in tandem with the test culture, under similar conditions, but without the presence of the test substance.

In one embodiment of the present invention, after appropriate incubation, total RNA is isolated from the cultured cells and prepared for Northern blot analysis as described in Example 1. After fractionation of mRNA on a 1% agarose formaldehyde gel using standard techniques, the mRNA is transferred to a membrane suitable for Northern

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blot analysis and hybridized with a ^{32}P -labeled OP-1 cDNA probe under conditions of high stringency. For example, the probe can be all or a portion of the murine OP-1 cDNA sequences described in Özkaynak, E., et al., Biochem. Biophys. Res. Commun., 30:116-123 (1991). Alternatively, the OP-1 cDNA probe can be all or a portion of the human OP-1 cDNA sequence (Özkaynak, E., et al., EMBO J., 9:2085-2093 (1990), deposited in the EMBL data library under accession number X51801).

Other OP-1 oligonucleotide probes can also be used in the Northern blot analysis. For example, a labeled OP-1 RNA oligonucleotide probe can be used. The probes can be of a length different from the cDNA probe used in Example 1. However, the probe must be of a length sufficient to specifically hybridize with OP-1 mRNA under conditions of high stringency. The probes can also vary in nucleotide sequence from the probe used in Example 1. However, the probe sequence must contain sufficient number of similar nucleotides to permit specific hybridization of the probe to OP-1 mRNA under conditions of high stringency.

The oligonucleotide probes used in this embodiment of the present invention can be labeled with radioactive molecules, such as ^{32}P . mRNA on the membrane which hybridized with the ^{32}P -labeled OP-1 oligonucleotide probe is then visualized by autoradiography, and quantified, for example, by laser scanning densitometry as described in Example 1. Alternatively, the probes can be labeled with non-radioactive molecules, such as peroxidase, biotin or digoxigenin. mRNA hybridized with non-radioactive OP-1 oligonucleotide probes is then visualized by color development by enzyme assay, binding to labeled streptavidin, or chemiluminescence.

After quantification, the amount of OP-1 mRNA expressed in the test culture (i.e., cells cultured in the presence of test substance) is compared to the amount of

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mRNA expressed in the control culture (i.e., cells cultured without the presence of the test culture). If the amount of OP-1 mRNA expressed in the test culture is higher than the amount of OP-1 mRNA expressed in the control culture, the presence of the test substance induced OP-1 mRNA expression, and thus, is capable of inducing bone formation. If the amount of OP-1 mRNA expressed in the test culture is less than the amount of mRNA expressed in the control culture, the presence of the test substance suppressed OP-1 mRNA expression, and thus, is capable of suppressing bone formation.

In another embodiment of the present invention, the synthesis of OP-1 protein is determined. Cultured cells can be prepared, denatured by SDS and heat treatment, and subjected to SDS polyacrylamide (PAGE) electrophoresis as described in Example 2. After separation on the basis of size, the separated proteins are transferred to a membrane suitable for Western blot analysis, and incubated with antibody specific for OP-1 protein (e.g., an OP-1 specific monoclonal, polyclonal antibody, or an antibody mix). Detection and quantification of OP-1 protein analyzed by Western blot analysis is performed as described in Example 2. Alternatively, OP-1 protein secreted into the culture medium can be measured by standard laboratory techniques (e.g., ELISA or RIA), also using antibodies specific for OP-1.

After quantification, the amount of OP-1 protein synthesized in the test culture (i.e., cells cultured in the presence of test substance) is compared to the amount of OP-1 protein synthesized in the control culture (i.e., cells cultured without the presence of the test culture). If the amount of OP-1 protein synthesized in the test culture is higher than the amount of OP-1 protein synthesized in the control culture, the presence of the test substance induced OP-1 protein synthesis, and thus,

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is capable of inducing bone formation. If the amount of OP-1 protein synthesized in the test culture is less than the amount of OP-1 protein synthesized in the control culture, the presence of the test substance suppressed OP-1 protein synthesis, and thus, is capable of suppressing bone formation.

The biological activity of the OP-1 protein synthesized, or secreted, can be measured using, for example, the in vivo bone matrix implant bioassay described in Rueger, D.C., et al., J. Biol. Chem., 265:13198-13205 (1990), the teachings of which are incorporated herein by reference. This assay measures the bone-inducing activity of OP-1 protein on bone matrix implanted in rats. Thus, if a test substance affects the biological activity of the OP-1 protein (e.g., by affecting maturation of the protein, or glycosylation of the protein) the increase, or decrease in biological activity can be determined.

Abnormalities in mineral and bone metabolism begin in early stages of loss of kidney function, and bone disease represents a major contributor to the morbidity of patients with end stage renal disease. Decreased renal synthesis of 1,25 dihydroxyvitamin D is clearly a major factor in the production of hyperparathyroid bone disease. However a subset of patients with renal osteodystrophy develop a low turnover bone disease not associated with aluminum or iron deposition (Salusky, I., et al., Miner. Electrolyte Metab., 17:237-280 (1991); Mathias, R., et al., J. Am. Soc. Nephrol., 3:1938-1946 (1993)). The etiology of this aplastic bone disease remains unknown. Thus, other factors produced by the kidney aside from activated vitamin D, may be necessary for adequate bone homeostasis.

Peptide hormones classically involved in bone metabolism could modulate OP-1 expression and production

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by the kidney via activation of the protein kinase A pathway, to stimulate the level of OP-1 mRNA expression. However, based on the finding that IMCD cells expressed OP-1 at such high levels, it is reasonable to believe that 5 renal OP-1 production may be an integral part of a mechanism distinct from parathyroid hormone (PTH) and vitamin D by which the kidney influences skeletal maturation and growth. The mIMCD-3 cell line, together with the assay described herein, are particularly useful 10 as a model system to study the pathway of bone morphogenesis regulated by OP-1. The IMCD cell line was developed from the terminal inner medullary collecting duct of a large T antigen SV40 mouse and it retains many characteristics of this nephron segment, including 15 increased transepithelial resistance in response to dexamethasone, inhibition of sodium flux by amiloride and atrial natriuretic peptide, and the ability to adapt to hypertonicity. Receptors to calcitriol or PTH have not been found in inner medulla. (Karashima, H., et al., 20 Kidney Int., 29:98 (1986); Alexander, E. and Schwartz, J., American J. of Kid. Dis., 18(5):612-618 (1991); Yoshitaka, M., et al., Am. J. Physiol., 263(32):F319-F327 (1992)). Therefore, it is reasonable to believe that alterations in acid-base status and/or mineralocorticoid effect might 25 modulate expression of OP-1 in this segment of the nephron. Thus, OP-1 protein may serve as a link between ion sensing cells in the inner medulla and cellular elements of the skeleton.

Substances identified in the method of the present 30 invention, found to affect OP-1 mRNA expression, or OP-1 protein synthesis, can be used to therapeutically treat a patient with bone disease, such as osteoporosis, to induce bone growth (bone morphogenesis). This method comprises administering to the patient an effective amount of a

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substance capable of inducing bone growth by inducing OP-1 mRNA expression, or OP-1 protein synthesis.

Administration of such bone-inducing substances can be by medically accepted techniques, including
5 intravenous, subcutaneous, or oral administration. Appropriate amounts, or effective dosages will, of course, vary from individual to individual and by type of disease and/or severity of the disease. Appropriate dosages can be calculated by those skilled in the art taking such
10 factors into account.

Conversely, the present invention provides a method of therapeutically treating a patient with bone disease, such as osteopetrosis, to suppress an increase in bone formation. This method comprises administering to the
15 patient an effective amount of a substance capable of suppressing bone growth by suppressing OP-1 mRNA expression, or OP-1 protein synthesis.

Of the extrarenal tissues studied, as described herein, ovary tissue had the highest OP-1 mRNA expression.
20 Thus, a possible role of the OP-1 synthesized by the ovary in the maintenance of bone homeostasis is also reasonable, especially in the relationship of age related OP-1 mRNA expression and OP-1 protein synthesis (e.g., post-menopausal osteoporosis). The method of the present
25 invention can also be used to identify substances capable of inducing bone formation by their effect on OP-1 mRNA expression, or OP-1 protein synthesis in ovary cells.

Additionally, IMCD cells of the kidney may make a unique form of OP-1 that does not cause calcification
30 immediately but would have to be processed to become active in calcification processes. Alternatively, IMCD OP-1 may have other functions unique to the kidney itself. Since IMCD cells secrete OP-1, these cells can be used in the described methods to further elucidate these
35 functions. Unique properties of IMCD-derived OP-1 would

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have considerable value. For example, because these cells are epithelial cells, it is reasonable to predict that OP-1 is only secreted on one side of the layer of cells, either the apical side (urine facing) or basolateral side (blood facing) of epithelial plasma membranes. Thus, the different forms of OP-1 could reflect one form of OP-1 going to one surface and the targeting of another form to the opposite surface.

It is also reasonable to predict that OP-1 secreted by renal epithelial cells is also present in the urine. Assay systems using IMCD cell supernatants can be designed to measure the urine and hence body stores, of OP-1 using OP-1 specific monoclonal antibodies such as 12G3. If renal IMCD cells make a specialized form of OP-1 this form could be distinguished from OP-1 made by other regions of the kidney as well as that filtered by glomeruli from the serum. Such an assay could distinguish between the various forms of OP-1. This information would be of value, for example, to distinguish which patients might benefit from treatment with OP-1 and/or stimulation of synthesis of OP-1 exogenous as opposed to patients requiring other therapy.

In addition to the role of OP-1 as a mediator of bone metabolism, OP-1 may also function as a "repair" molecule in mammalian kidney tissue. As discussed above, it has been demonstrated that OP-1 mRNA and protein expression is 2 fold higher in rat kidney as compared to 5 other tissues. Comparison of the OP-1 expression in the same 6 tissues in young (4 week old) versus old (2 year old) rats demonstrated a significant decline in renal medullary OP-1 expression as a function of age. In addition, both in situ hybridization and immunocytochemistry studies showed that OP-1 is expressed by glomerular tufts and adjacent vessels in the renal cortex as well as tubules and vasculature in the kidney medulla. (J. Amer. Soc.

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Nephrol., 4:700 Abstracts 83P and 23P (1993); Paredes, A.,
et al., J. Amer. Soc. Nephrol., 5:855 Abstract 68P
(1994)).

A similar pattern of OP-1 has now been demonstrated
5 by staining human kidney biopsy samples using identical
antibody probes (the monoclonal antibody, 12G3). In
tissue biopsy sections of both rat and human kidneys, the
distribution of OP-1 among renal tubules is not
homogenous. In selected renal tubules, there is abundant
10 OP-1 protein, whereas tubules immediately adjacent have
little, or no detectable OP-1 expression. These data
suggest that the observed heterogeneity in detectable OP-1
protein may be due to the fact that some tubules are
undergoing remodeling or repair, whereas others are
15 quiescent.

Moreover, described herein for the first time,
examination of a limited number of (n=4) of kidney biopsy
sections from children with renal dysplasia revealed
abundant OP-1 staining with the OP-1 specific antibody,
20 12G3. The overall pattern of OP-1 staining also included
primarily the tubular epithelial cells and the adjacent
interstitium. These data reasonably suggest that the
amount of OP-1 protein detected is indicative of the
amount of remodeling of diseased kidney tissue thus,
25 supporting the paracrine role of OP-1 protein as a repair
molecule.

Other reports provide support for the role of OP-1 as
a modulator of tissue remodeling. Studies have shown that
OP-1 appears to act as a potent renal morphogen in the
30 developing kidney. When assayed in an in vitro organ
culture system consisting of explants of mouse metanephric
blastema cultured in a serum free media, OP-1 dramatically
altered tubular development and gave rise to a new cell
type that possesses many characteristics of chondrocytes
35 (Avenier, E.D., J. Amer. Soc. Nephrol., 3:461 Abstract 82P

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(1993)). OP-1 is also highly expressed in embryonic tissues (Ozkaynak, E., et al., EMBO J., 9:2085-2093 (1992)) and in neuronal cells in tissue culture alters the patterns of aggregation and development of these cells in a highly specific fashion (Perides, G. et al., Proc. Natl. Acad. Sci., 89:10326-10330 (1992)).

In addition, studies of unrelated proteins have provided examples of proteins synthesized by the kidney that have both autocrine (acting at a distance) and paracrine (acting locally) effects. Several proteins derived from the kidney have been demonstrated to possess effects on other organs as well as serving to modulate cellular growth within the kidney. Renin/angiotensin proteins alter systemic blood pressure and acts as a factor for kidney growth (Ichikawa, I. and Harris, R.C., Kidney International, 40:583-596 (1991)). Endothelin, produced by kidney endothelial cells and exerts both local and systemic vasoconstrictive effects and effects proliferation of glomerular endothelial cells (Kon, V. and K. Bodi, Kidney International, 40:1-12 (1991)). Epidermal growth factor (EGF) is produced largely by epithelial cells of the thick ascending limb of Henle, but EGF receptors are present in all epithelial cells of the kidney where it stimulates tubular proliferation (Fine, L.J., Amer. Soc. of Nephrol., 2:1163-1170 (1992)).

Most renal biopsies are performed in an effort to prognosticate the outcome, as well as determine the cause of a patient's renal disease. Examination of OP-1 expression using antisera in these renal biopsies can be used to assess the kidney's response or lack thereof to a specific renal injury. Such a test can also be useful to determine specific diseases where OP-1 might play a significant fold in its pathogenesis. Lastly, since epithelial cells are shed into the urine and, as discussed above, it has been demonstrated the OP-1 is secreted from

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renal epithelial cells, OP-1 antisera can be useful to determine the level of OP-1 expression in urine samples from patients with renal disease.

Thus, as a result of Applicants' work, a model assay system is now available to identify substances capable of inducing new bone formation by the evaluation of the effect of test substances on the expression of OP-1 mRNA expression and OP-1 protein synthesis in inner medullary collecting duct cells. Significant levels of OP-1 mRNA transcripts and proteins are present in the renal medulla. Therefore, renal OP-1 production in the inner medulla may act as an alternative pathway to the classic PTH and vitamin D mechanisms by which kidney modulates bone homeostasis. Moreover, based on additional data presented herein, OP-1 may also act in the repair of normal and abnormal kidney tissue.

The invention is further illustrated by the following specific examples.

Example 1: Northern Blot Analysis to Detect OP-1 mRNA
20 Expression

Animals: Young adult female Sprague-Dawley rats approximately 300 gms (2-4 months old) were purchased from Charles River Laboratories (Wilmington, MA). After euthanasia under general anesthesia, organs were flushed with ice-cold saline via intracardiac puncture. Renal cortex, medulla and papilla were immediately dissected. Equal amounts of lung, liver, brain, small intestine and ovary were also extracted.

Cultured Cells: The following cell lines were surveyed:
30 opossum kidney (OK) cells, a gift from Dr. M.H. Montrose, Johns Hopkins University, MD; Madin Darby Conni Kidney (MDCK) cells obtained from American Type Culture

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Collection (Rockville, MD); and inner medullary collecting duct (IMCD) cells derived from a transgenic SV-40 mouse (Rauchman, M.I., *et al.*, Am. J. Physiol., 265-271 (1993)) were obtained from Dr. M. Ziedel, Brigham and Women's Hospital, Boston, MA. The cells were grown to confluency on tissue culture dishes (Nunclon Delta; Nunc, Inc; Naperville, IL) at pH 7.4 in incubators at 37°C with a 5% CO₂, 95% air atmosphere. OK and IMCD cells were fed on alternate days in Dulbecco's Modified Eagle's Medium (DMEM)-F12 (GIBCO, Gaithersburg, MD) supplemented with 29 mM bicarbonate, 2mM glutamate, 100 IU/cc penicillin, 100 pg/cc of streptomycin and 10% (OK cells) or 5% (IMCD cells) fetal calf serum. MDCK cells were fed on alternate days with a standard MEM culture medium (GIBCO, Gaithersburg, MD) supplemented with Earles Salt, 2 mM glutamate, 10% fetal calf serum, 100 IU/cc penicillin and 100 pg/cc of streptomycin.

Probes: A 680 bp fragment (SEQ ID NO: 1) containing two thirds of the pro domain and one third of the mature murine OP-1 cDNA (nucleotides 370-1050 immediately upstream to the highly conserved 7-cysteine domain present in all members of the TGF- β superfamily) was used as a probe for Northern analysis. (Özkaynak, E., *et al.*, Biochem. Biophys. Res. Commun., 30:116-123 (1991)). USB Sequenase Random Prime DNA labeling Kit (United States Biochemical, Cleveland, OH) and $\alpha^{32}\text{P}$ dCTP (New England Nuclear, Boston, MA) were used for standard labeling reactions. A 40 bp oligonucleotide of mouse β -actin (Oncogene Science, Uniondale, NY) was used as control gene.

RNA Preparation: Total RNA was isolated from animal tissues (100 mg/assay) and cultured cells (90-100% confluency, 100 x 20 mm plate/assay) using the guanidine

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isothiocyanate/phenol-chloroform method (Chomczynski, P. and Sacchi, N., Anal. Biochem., 162:156-159 (1987)). Poly A⁺ was selected by oligo dT cellulose using the mRNA MicroFastTrack Kit from Invitrogen, San Diego, CA.

5 Northern Blot Analysis: Equal amounts of mRNA were fractionated on a 1% agarose formaldehyde gel using standard techniques. RNA was transferred to Duralon -UV membranes (Stratagene, La Jolla, CA) by capillary blotting with 10x standard sodium citrate (SSC). The membranes were
10 then baked at 80°C under vacuum for 2 hours. After this treatment, membranes were prehybridized with 50% deionized Formamide (Sigma), 10% Dextran Sulfate, 1% sodium dodecyl sulfate (SDS), 1 M NaCl and 100 pg/ml of denatured sonicated salmon sperm DNA (Sigma) for 2 hours at 42°C in
15 a hybridization oven. Using the same solution, the membranes were then hybridized for 20 hours at 42°C with 1×10^6 cpm/ml of the ³²P-labeled OP-1 cDNA probe. Membranes were washed for 30 minutes with 0.1x SSC-0.1x SDS at 65°C and then subjected to autoradiography at -80°C for 24
20 hours using Kodak XAR-5 film and an intensifying screen (Lightning Plus; Eastman Kodak, Rochester, NY). To control for mRNA content and quality, the blots were stripped and subsequently hybridized with mouse β -actin. The intensity of the signal was quantified by laser scanning
25 densitometer (model SL-504-XL; Biomed Instruments, Fullerton, CA). The ratio OP-1 mouse β -actin signal was calculated for each tissue and the highest value within an animal was assigned as 100%. The percentages of mRNA expression for the rest of the tissues were figured out by
30 comparison of their ratios to the highest one.

Statistical analysis: Data are expressed as means \pm SEM. Since the variables were not normally distributed, OP-1 mRNA expression among tissues was statistically analyzed

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by Man Whitney non parametric tests. Statistical significance was defined as $P < 0.05$.

Example 2: Western Blot Analysis of OP-1 Protein Synthesis

Generation of the Anti-OP-1 Antibody 12G3

5 The selection of the IgG₁ monoclonal anti-OP-1 antibody 12G3 was accomplished by injecting recombinant human OP-01 and fusing the spleen cells with murine myeloma cells, followed by screening using Western blots of oxidized and reduced OP-1 (Vukicevic, S., et al.,
10 Biochem. Biophys. Res. Commun. 198, 693-700 (1994))

 The injected recombinant human OP-1 used in the generation of 12G3 was composed of amino acids 293-431 where amino terminal methionine of pre-pro OP-1 is defined as residue #1 (Özkaynak, E., et al., EMBO J. 9, 2085-2093
15 (1993)).

Gel Electrophoresis and Immunoblotting: Tissue samples were minced and denatured by rapid addition of 10% SDS in a 1:1 dilution and heating to 100° C for 5 minutes. The samples were then stored at -80° C after determination of
20 their protein content using the BCA protein assay (Pierce Chemical Co., Rockford, IL). Forty micrograms of protein from each sample were separated by SDS-PAGE using a Mighty Small apparatus, (Hoefer Scientific Instruments, San Francisco, CA) (Laemmli, U.K., Nature London, 277:680-685
25 (1980)). After SDS-PAGE, the proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) using a solution of 25 mM Tris, 200 mM glycine, and 20% methanol. These membranes were then removed from the transfer
30 immunoblotted with monoclonal antibody 12G3 after blocking with 5% bovine serum albumin (Sigma Chem. Co., St. Louis, MO) dissolved in water. The blots were incubated

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overnight using 12 µg/ml of 12G3 diluted in 50 mM NaPO₄, pH 7.4, 100 mM NaCl phosphate buffered saline (PBS) with 0.01% Tween-20 (PBS-Tween). After incubation with monoclonal 12G3, the blots were rinsed repeatedly with Tween-PBS and bound immunoglobulin was detected using affinity purified peroxidase conjugated goat antimouse antisera (Organon Teknika, West Chester, PA) and chemiluminescence (ECL Amersham Life Sci., Arlington, IL).

Example 3: Immunocytochemical Analysis to Detect the Presence of OP-1 Protein

Immunocytochemistry (IC): Following an overnight incubation in 3% normal goat serum at 4° C, rat kidney sections were incubated in primary monoclonal antibody 12G3, that was diluted in PBS, 1% fetal calf serum and 0.1% sodium azide for 60 minutes at room temperature. Subsequently sections were exposed to a peroxidase-conjugated goat anti-mouse immunoglobulin (Jackson ImmunoResearch, Westgrove, PA) in the same diluent, at a dilution of 1:40 for an additional 60 minutes. The slides received sequential washes of 5 minutes duration in PBS then PBS containing 0.2% gelatin after each incubation. After the above series of incubations, the specimens were exposed to 5 minute intervals of 0.1 M sodium acetate buffer (pH 5.2) and 0.25 mg/ml 3-amino 9-ethylcarbazole (AEC) (Sigma Chem. Co., St. Louis, MO) in 2% N-N dimethylformamide (Sigma Chem. Co., St. Louis, MO) followed by 0.1 M sodium acetate buffer (pH 5.2) with 0.03% hydrogen peroxide. The specimens were finally washed in tap water for 5 minutes, counterstained with hematoxylin, and mounted in glycerol gelatin (Sigma Chem. Co., St. Louis, MO).

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In Situ Hybridization: A ^{32}P -labelled antisense RNA probe was prepared by linearizing Bluescript (Stratagene, La Jolla, CA) containing the 680 bp OP-1 cDNA with Hind III followed by transcription with T7 polymerase (Promega Inc., Madison, WI) in the presence of [^{32}P] CTP (800 Ci/mmol.). As a control, ^{32}P -labelled sense RNA probe was prepared by linearizing the same vector with BAM H1 followed by transcription with T3 polymerase under identical conditions.

10 Paraffin sections of adult C57BL/6 mouse kidney were subjected to *in situ* hybridization as described in Arceci, R.J., et al., Proc. Natl. Acad. Sci. USA, 86:8818-8822 (1989) using [^{32}P] CTP-labeled sense (Control) or antisense (Experimental) RNA probes transcribed from
15 Bluescript-OP-1 vector as described above. After autoradiography, the slides were stained with hematoxylin/eosin prior to photographic analysis.

Equivalents

Those skilled in the art will recognize, or be able
20 to ascertain using no more than routine experimentation, many equivalents to specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Children's Medical Center Corporation
300 Longwood Avenue
Boston, Massachusetts 02115
- (ii) TITLE OF INVENTION: METHOD OF IDENTIFYING A SUBSTANCE
CAPABLE OF INDUCING BONE FORMATION
- (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Hamilton, Brook, Smith & Reynolds, P.C.
 - (B) STREET: Two Militia Drive
 - (C) CITY: Lexington
 - (D) STATE: Massachusetts
 - (E) COUNTRY: USA
 - (F) ZIP: 02173-4799
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/153,183
 - (B) FILING DATE: 16-NOV-1993
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Brook, David E.
 - (B) REGISTRATION NUMBER: 22,592
 - (C) REFERENCE/DOCKET NUMBER: CMCC-349
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 617-861-6240
 - (B) TELEFAX: 617-861-9540

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1872 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown
- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 104..1390

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCAGCAAG TGACCTCGGG TCGTGACC CGTCCCTGCC CCCTCCGCTG CCACCTGGGG	60
CGGCGCGGGC CCGGTGCCCC GGATCGCGCG TAGAGCCGGC GCG ATG CAC GTG CGC	115
Met His Val Arg	
1	
TCG CTG CGC GCT GCG GCG CCA CAC AGC TTC GTG GCG CTC TGG GCG CCT	163
Ser Leu Arg Ala Ala Ala Pro His Ser Phe Val Ala Leu Trp Ala Pro	
5 10 15 20	
CTG TTC TTG CTG CGC TCC GCC CTG GCC GAT TTC AGC CTG GAC AAC GAG	211
Leu Phe Leu Leu Arg Ser Ala Leu Ala Asp Phe Ser Leu Asp Asn Glu	
25 30 35	
GTG CAC TCC AGC TTC ATC CAC CGG CGC CTC CGC AGC CAG GAG CGG CGG	259
Val His Ser Ser Phe Ile His Arg Arg Leu Arg Ser Gln Glu Arg Arg	
40 45 50	
GAG ATG CAG CGG GAG ATC CTG TCC ATC TTA GGG TTG CCC CAT CGC CCG	307
Glu Met Gln Arg Glu Ile Leu Ser Ile Leu Gly Leu Pro His Arg Pro	
55 60 65	
CGC CCG CAC CTC CAG GGA AAG CAT AAT TCG GCG CCC ATG TTC ATG TTG	355
Arg Pro His Leu Gln Gly Lys His Asn Ser Ala Pro Met Phe Met Leu	
70 75 80	
GAC CTG TAC AAC GCC ATG GCG GTG GAG GAG AGC GGG CCG GAC GGA CAG	403
Asp Leu Tyr Asn Ala Met Ala Val Glu Glu Ser Gly Pro Asp Gly Gln	
85 90 95 100	
GGC TTC TCC TAC CCC TAC AAG GCC GTC TTC AGT ACC CAG GGC CCC CCT	451
Gly Phe Ser Tyr Pro Tyr Lys Ala Val Phe Ser Thr Gln Gly Pro Pro	
105 110 115	
TTA GCC AGC CTG CAG GAC AGC CAC TTC CTC ACT GAC GCC GAC ATG GTC	499
Leu Ala Ser Leu Gln Asp Ser His Phe Leu Thr Asp Ala Asp Met Val	
120 125 130	
ATG AGC TTC GTC AAC CTA GTG GAA CAT GAC AAA GAA TTC TTC CAC CCT	547
Met Ser Phe Val Asn Leu Val Glu His Asp Lys Glu Phe Phe His Pro	
135 140 145	
CGA TAC CAC CAT CGG GAG TTC CGG TTT GAT CTT TCC AAG ATC CCC GAG	595
Arg Tyr His His Arg Glu Phe Arg Phe Asp Leu Ser Lys Ile Pro Glu	
150 155 160	
GGC GAA CGG GTG ACC GCA GCC GAA TTC AGG ATC TAT AAG GAC TAC ATC	643
Gly Glu Arg Val Thr Ala Ala Glu Phe Arg Ile Tyr Lys Asp Tyr Ile	
165 170 175 180	
CGG GAG CGA TTT GAC AAC GAG ACC TTC CAG ATC ACA GTC TAT CAG GTG	691
Arg Glu Arg Phe Asp Asn Glu Thr Phe Gln Ile Thr Val Tyr Gln Val	
185 190 195	
CTC CAG GAG CAC TCA GGC AGG GAG TCG GAC CTC TTC TTG CTG GAC AGC	739
Leu Gln Glu His Ser Gly Arg Glu Ser Asp Leu Phe Leu Asp Ser	
200 205 210	

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CGC ACC ATC TGG GCT TCT GAG GAG GGC TGG TTG GTG TTT GAT ATC ACA	787
Arg Thr Ile Trp Ala Ser Glu Glu Gly Trp Leu Val Phe Asp Ile Thr	
215 220 225	
GCC ACC AGC AAC CAC TGG GTG GTC AAC CCT CGG CAC AAC CTG GGC TTA	835
Ala Thr Ser Asn His Trp Val Asn Pro Arg His Asn Leu Gly Leu	
230 235 240	
CAG CTC TCT GTG GAG ACC CTG GAT GGG CAG AGC ATC AAC CCC AAG TTG	883
Gln Leu Ser Val Glu Thr Leu Asp Gly Gln Ser Ile Asn Pro Lys Leu	
245 250 255 260	
GCA GGC CTG ATT GGA CGG CAT GGA CCC CAG AAC AAG CAA CCC TTC ATG	931
Ala Gly Leu Ile Gly Arg His Gly Pro Gln Asn Lys Gln Pro Phe Met	
265 270 275	
GTG GCC TTC TTC AAG GCC ACG GAA GTC CAT CTC CGT AGT ATC CGG TCC	979
Val Ala Phe Lys Ala Thr Glu Val His Leu Arg Ser Ile Arg Ser	
280 285 290	
ACG GGG GGC AAG CAG CGC AGC CAG AAT CGC TCC AAG ACG CCA AAG AAC	1027
Thr Gly Gly Lys Gln Arg Ser Gln Asn Arg Ser Lys Thr Pro Lys Asn	
295 300 305	
CAA GAG GCC CTG AGG ATG GCC AGT GTG GCA GAA AAC AGC AGC AGT GAC	1075
Gln Glu Ala Leu Arg Met Ala Ser Val Ala Glu Asn Ser Ser Ser Asp	
310 315 320	
CAG AGG CAG GCC TGC AAG AAA CAT GAG CTG TAC GTC AGC TTC CGA GAC	1123
Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr Val Ser Phe Arg Asp	
325 330 335 340	
CTT GGC TGG CAG GAC TGG ATC ATT GCA CCT GAA GGC TAT GCT GCC TAC	1171
Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu Gly Tyr Ala Ala Tyr	
345 350 355	
TAC TGT GAG GGA GAG TGC GCC TTC CCT CTG AAC TCC TAC ATG AAC GCC	1219
Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn Ser Tyr Met Asn Ala	
360 365 370	
ACC AAC CAC GCC ATC GTC CAG ACA CTG GTT CAC TTC ATC AAC CCA GAC	1267
Thr Asn His Ala Ile Val Gln Thr Leu Val His Phe Ile Asn Pro Asp	
375 380 385	
ACA GTA CCC AAG CCC TGC TGT GCG CCC ACC CAG CTC AAC GCC ATC TCT	1315
Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln Leu Asn Ala Ile Ser	
390 395 400	
GTC CTC TAC TTC GAC GAC AGC TCT AAT GTC ATC CTG AAG AAG TAC AGA	1363
Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile Leu Lys Lys Tyr Arg	
405 410 415 420	
AAC ATG GTG GTC CGG GCC TGT GGC TGC CACTAGCTCT TCCTGAGACC	1410
Asn Met Val Val Arg Ala Cys Gly Cys	
425	
CTGACCTTTG CGGGGCCACA CCTTTCCAAA TCTTCGATGT CTCACCATCT AAGTCTCTCA	1470
CTGCCACCT TGGCGAGGAG CCAACAGACC AACCTCTCCT GAGCCTTCCC CTCACCTCCC	1530
CAACCGGAAG CATGTAAGGG TTCCAGAAAC CTGAGCGTGC AGGCAGCTGA TGAGCGCCCT	1590

-34-

TTCCTTCTGG CACGTGACGG ACAAGATCCT ACCAGCTACC ACAGCAAACG CCTAAGAGCA 1650
 GGAAAAATGT CTGCCAGGAA AGTGTCCATT GGCCACATGG CCCCTGGCGC TCTGAGTCTT 1710
 TGAGGAGTAA TCGCAAGCCT CGTTCAGCTG CAGCAGAAGG AAGGGCTTAG CCAGGGTGGG 1770
 CGCTGGCGTC TGTGTTGAAG GGAAACCAAG CAGAAGCCAC TGTAATGATA TGTCACAATA 1830
 AAACCCATGA ATGAAAAAAAA AAAAAAAAAA AAAAAAAAAA AA 1872

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 429 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met His Val Arg Ser Leu Arg Ala Ala Ala Pro His Ser Phe Val Ala
 1 5 10 15
 Leu Trp Ala Pro Leu Phe Leu Leu Arg Ser Ala Leu Ala Asp Phe Ser
 20 25 30
 Leu Asp Asn Glu Val His Ser Ser Phe Ile His Arg Arg Leu Arg Ser
 35 40 45
 Gln Glu Arg Arg Glu Met Gln Arg Glu Ile Leu Ser Ile Leu Gly Leu
 50 55 60
 Pro His Arg Pro Arg Pro His Leu Gln Gly Lys His Asn Ser Ala Pro
 65 70 75 80
 Met Phe Met Leu Asp Leu Tyr Asn Ala Met Ala Val Glu Glu Ser Gly
 85 90 95
 Pro Asp Gly Gln Gly Phe Ser Tyr Pro Tyr Lys Ala Val Phe Ser Thr
 100 105 110
 Gln Gly Pro Pro Leu Ala Ser Leu Gln Asp Ser His Phe Leu Thr Asp
 115 120 125
 Ala Asp Met Val Met Ser Phe Val Asn Leu Val Glu His Asp Lys Glu
 130 135 140
 Phe Phe His Pro Arg Tyr His His Arg Glu Phe Arg Phe Asp Leu Ser
 145 150 155 160
 Lys Ile Pro Glu Gly Glu Arg Val Thr Ala Ala Glu Phe Arg Ile Tyr
 165 170 175
 Lys Asp Tyr Ile Arg Glu Arg Phe Asp Asn Glu Thr Phe Gln Ile Thr
 180 185 190
 Val Tyr Gln Val Leu Gln Glu His Ser Gly Arg Glu Ser Asp Leu Phe
 195 200 205

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Leu Leu Asp Ser Arg Thr Ile Trp Ala Ser Glu Glu Gly Trp Leu Val
 210 215 220
 Phe Asp Ile Thr Ala Thr Ser Asn His Trp Val Val Asn Pro Arg His
 225 230 235 240
 Asn Leu Gly Leu Gln Leu Ser Val Glu Thr Leu Asp Gly Gln Ser Ile
 245 250 255
 Asn Pro Lys Leu Ala Gly Leu Ile Gly Arg His Gly Pro Gln Asn Lys
 260 265 270
 Gln Pro Phe Met Val Ala Phe Phe Lys Ala Thr Glu Val His Leu Arg
 275 280 285
 Ser Ile Arg Ser Thr Gly Gly Lys Gln Arg Ser Gln Asn Arg Ser Lys
 290 295 300
 Thr Pro Lys Asn Gln Glu Ala Leu Arg Met Ala Ser Val Ala Glu Asn
 305 310 315 320
 Ser Ser Ser Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr Val
 325 330 335
 Ser Phe Arg Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu Gly
 340 345 350
 Tyr Ala Ala Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn Ser
 355 360 365
 Tyr Met Asn Ala Thr Asn His Ala Ile Val Gln Thr Leu Val His Phe
 370 375 380
 Ile Asn Pro Asp Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln Leu
 385 390 395 400
 Asn Ala Ile Ser Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile Leu
 405 410 415
 Lys Lys Tyr Arg Asn Met Val Val Arg Ala Cys Gly Cys
 420 425

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CLAIMS

The invention claimed is:

1. A method of identifying a substance capable of inducing new bone formation comprising determining
5 the effect the substance has on the expression of OP-1 mRNA in mammalian cells.
2. A method of identifying a substance capable of inducing new bone formation comprising determining the effect the substance has on inducing the
10 expression of OP-1 protein in mammalian cells.
3. A method of identifying a substance capable of inducing new bone formation, comprising determining the effect the substance has on inducing the expression of OP-1 mRNA expression in mammalian
15 cells, comprising the steps of:
 - a) growing mammalian cells in culture;
 - b) introducing into the cell culture of step a) a test substance in an amount sufficient to induce OP-1 mRNA expression if the substance possesses
20 inducing properties;
 - c) isolating mRNA from the cultured cells of step b) and determining the amount of OP-1 mRNA expressed by the cultured cells; and
 - d) comparing the amount of OP-1 mRNA expressed by
25 the cultured cells of step b) to the amount of OP-1 mRNA expressed by cultured cells under similar conditions but without the presence of the test substance to determine the effectiveness of the test substance to induce
30 OP-1 mRNA expression.

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4. A method of Claim 3 wherein the mammalian cells are kidney cells.
5. A method of Claim 4 wherein the mammalian kidney cells are inner medullary collecting duct cells.
- 5 6. A method of Claim 5 wherein the inner medullary collecting duct cells are murine cell line mIMCD-3 cells.
7. A method of Claim 3 wherein the mammalian cells are ovarian cells.
- 10 8. A method of Claim 3 wherein the expression of OP-1 mRNA is determined by Northern blot analysis using labeled OP-1 cDNA as a probe.
9. A substance identified by the method of Claim 3 which induces OP-1 mRNA expression.
- 15 10. A method of identifying a substance capable of inducing new bone formation, comprising determining the effect the substance has on inducing the synthesis of OP-1 protein in mammalian cells, comprising the steps of:
 - 20 a) growing mammalian cells in culture;
 - b) introducing into the cell culture of step a) a test substance in an amount sufficient to induce OP-1 protein synthesis if the substance possesses inducing properties;
 - 25 c) determining the amount of OP-1 protein synthesized by the cultured cells of step b);and

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- d) comparing the amount of OP-1 protein synthesized by the cultured cells of step b) to the amount of OP-1 protein synthesized by cultured cells under similar conditions but without the presence of the test substance to determine the effectiveness of the test substance to induce OP-1 protein synthesis.
- 5
11. A method of Claim 10 wherein the mammalian cells are kidney cells.
- 10 12. A method of Claim 11 wherein the mammalian kidney cells are inner medullary collecting duct cells.
13. A method of Claim 12 wherein the inner medullary collecting duct cells are murine cell line mIMCD-3 cells.
- 15 14. A method of Claim 10 wherein the mammalian cells are ovarian cells.
15. A method of Claim 10 wherein the synthesis of OP-1 protein is measured by Western blot analysis using antibody reactive with OP-1 protein.
- 20 16. A substance identified by the method of Claim 10 which induces OP-1 protein synthesis.
17. A method of treating bone disease in an individual comprising administering to the individual an effective amount of a substance which increases OP-1 mRNA expression.
- 25

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18. A method of treating bone disease in an individual comprising administering to the individual an effective amount of a substance which increase OP-1 protein synthesis.
- 5 19. A substance capable of inducing bone formation by inducing OP-1 mRNA expression in inner medullary collecting ducts cells.
20. A substance capable of inducing bone formation by inducing OP-1 protein synthesis in inner medullary
10 collecting duct cells.
21. A method of evaluating the extent of renal dysplasia in a mammal with renal disease comprising detecting the presence of OP-1 protein in the kidney of the mammal with renal disease comprising the steps of:
15 a) obtaining a kidney tissue biopsy sample from a mammal with renal disease;
b) contacting the kidney biopsy sample with an antibody specific for OP-1 protein and
20 maintaining the biopsy sample in contact with the antibody under conditions suitable for the antibody to bind to OP-1 protein present in the kidney tissue;
c) detecting the amount of antibody specifically bound to OP-1 protein present in the biopsy
25 sample; and
d) comparing the amount of OP-1 protein present in the kidney biopsy sample obtained from the mammal with renal disease to the amount of OP-1 protein present in a kidney biopsy sample
30 obtained from a mammal without renal disease, wherein the amount of OP-1 protein detected is an indication of the extent of renal dysplasia.

CTGCAGCAAG TGACCTCGGG TCGTGGACCG CTGCCCTGCC CCCTCCGCTG CCACCTGGGG	60
CGGCGCGGGC CCGGTGCCCC GGATCGCGCG TAGAGCCGGC GCG ATG CAC GTG CGC	115
Met His Val Arg	
1	
TCG CTG CGC GCT GCG GCG CCA CAC AGC TTC GTG GCG CTC TGG GCG CCT	163
Ser Leu Arg Ala Ala Ala Pro His Ser Phe Val Ala Leu Trp Ala Pro	
5 10 15 20	
CTG TTC TTG CTG CGC TCC GCC CTG GCC GAT TTC AGC CTG GAC AAC GAG	211
Leu Phe Leu Leu Arg Ser Ala Leu Ala Asp Phe Ser Leu Asp Asn Glu	
25 30 35	
GTG CAC TCC AGC TTC ATC CAC CGG CGC CTC CGC AGC CAG GAG CGG CGG	259
Val His Ser Ser Phe Ile His Arg Arg Leu Arg Ser Gln Glu Arg Arg	
40 45 50	
GAG ATG CAG CGG GAG ATC CTG TCC ATC TTA GGG TTG CCC CAT CGC CCG	307
Glu Met Gln Arg Glu Ile Leu Ser Ile Leu Gly Leu Pro His Arg Pro	
55 60 65	
CGC CCG CAC CTC CAG GGA AAG CAT AAT TCG GCG CCC ATG TTC ATG TTG	355
Arg Pro His Leu Gln Gly Lys His Asn Ser Ala Pro Met Phe Met Leu	
70 75 80	
GAC CTG TAC AAC GCC ATG GCG GTG GAG GAG AGC GGG CCG GAC GGA CAG	403
Asp Leu Tyr Asn Ala Met Ala Val Glu Glu Ser Gly Pro Asp Gly Gln	
85 90 95 100	
GGC TTC TCC TAC CCC TAC AAG GCC GTC TTC AGT ACC CAG GGC CCC CCT	451
Gly Phe Ser Tyr Pro Tyr Lys Ala Val Phe Ser Thr Gln Gly Pro Pro	
105 110 115	
TTA GCC AGC CTG CAG GAC AGC CAC TTC CTC ACT GAC GCC GAC ATG GTC	499
Leu Ala Ser Leu Gln Asp Ser His Phe Leu Thr Asp Ala Asp Met Val	
120 125 130	
ATG AGC TTC GTC AAC CTA GTG GAA CAT GAC AAA GAA TTC TTC CAC CCT	547
Met Ser Phe Val Asn Leu Val Glu His Asp Lys Glu Phe Phe His Pro	
135 140 145	
CGA TAC CAC CAT CGG GAG TTC CGG TTT GAT CTT TCC AAG ATC CCC GAG	595
Arg Tyr His His Arg Glu Phe Arg Phe Asp Leu Ser Lys Ile Pro Glu	
150 155 160	
GGC GAA CGG GTG ACC GCA GCC GAA TTC AGG ATC TAT AAG GAC TAC ATC	643
Gly Glu Arg Val Thr Ala Ala Glu Phe Arg Ile Tyr Lys Asp Tyr Ile	
165 170 175 180	
CGG GAG CGA TTT GAC AAC GAG ACC TTC CAG ATC ACA GTC TAT CAG GTG	691
Arg Glu Arg Phe Asp Asn Glu Thr Phe Gln Ile Thr Val Tyr Gln Val	
185 190 195	
CTC CAG GAG CAC TCA GGC AGG GAG TCG GAC CTC TTC TTG CTG GAC AGC	739
Leu Gln Glu His Ser Gly Arg Glu Ser Asp Leu Phe Leu Leu Asp Ser	
200 205 210	
CGC ACC ATC TGG GCT TCT GAG GAG GGC TGG TTG GTG TTT GAT ATC ACA	787
Arg Thr Ile Trp Ala Ser Glu Glu Gly Trp Leu Val Phe Asp Ile Thr	
215 220 225	
GCC ACC AGC AAC CAC TGG GTG GTC AAC CCT CGG CAC AAC CTG GGC TTA	835
Ala Thr Ser Asn His Trp Val Val Asn Pro Arg His Asn Leu Gly Leu	
230 235 240	

FIGURE 1A

SUBSTITUTE SHEET (RULE 26)

CAG CTC TCT GTG GAG ACC CTG GAT GGG CAG AGC ATC AAC CCC AAG TTG Gln Leu Ser Val Glu Thr Leu Asp Gly Gln Ser Ile Asn Pro Lys Leu 245 250 255 260	883
GCA GGC CTG ATT GGA CGG CAT GGA CCC CAG AAC AAG CAA CCC TTC ATG Ala Gly Leu Ile Gly Arg His Gly Pro Gln Asn Lys Gln Pro Phe Met 265 270 275	931
GTG GCC TTC TTC AAG GCC ACG GAA GTC CAT CTC CGT AGT ATC CGG TCC Val Ala Phe Phe Lys Ala Thr Glu Val His Leu Arg Ser Ile Arg Ser 280 285 290	979
ACG GGG GGC AAG CAG CGC AGC CAG AAT CGC TCC AAG ACG CCA AAG AAC Thr Gly Gly Lys Lys Gln Arg Ser Gln Asn Arg Ser Lys Thr Pro Lys Asn 295 300 305	1027
CAA GAG GCC CTG AGG ATG GCC AGT GTG GCA GAA AAC AGC AGC AGT GAC Gln Glu Ala Leu Arg Met Ala Ser Val Ala Glu Asn Ser Ser Ser Asp 310 315 320	1075
CAG AGG CAG GCC TGC AAG AAA CAT GAG CTG TAC GTC AGC TTC CGA GAC Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr Val Ser Phe Arg Asp 325 330 335 340	1123
CTT GGC TGG CAG GAC TGG ATC ATT GCA CCT GAA GGC TAT GCT GCC TAC Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu Gly Tyr Ala Ala Tyr 345 350 355	1171
TAC TGT GAG GGA GAG TGC GCC TTC CCT CTG AAC TCC TAC ATG AAC GCC Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn Ser Tyr Met Asn Ala 360 365 370	1219
ACC AAC CAC GCC ATC GTC CAG ACA CTG GTT CAC TTC ATC AAC CCA GAC Thr Asn His Ala Ile Val Gln Thr Leu Val His Phe Ile Asn Pro Asp 375 380 385	1267
ACA GTA CCC AAG CCC TGC TGT GCG CCC ACC CAG CTC AAC GCC ATC TCT Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln Leu Asn Ala Ile Ser 390 395 400	1315
GTC CTC TAC TTC GAC GAC AGC TCT AAT GTC ATC CTG AAG AAG TAC AGA Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile Leu Lys Lys Tyr Arg 405 410 415 420	1363
AAC ATG GTG GTC CGG GCC TGT GGC TGC CACTAGCTCT TCCTGAGACC Asn Met Val Val Arg Ala Cys Gly Cys 425	1410
CTGACCTTTG CGGGGCCACA CCTTTCCAAA TCTTCGATGT CTCACCATCT AAGTCTCTCA	1470
CTGCCACCT TGGCGAGGAG CCAACAGACC AACCTCTCCT GAGCCTTCCC CTCACCTCCC	1530
CAACCGGAAG CATGTAAGGG TTCCAGAAAC CTGAGCGTGC AGGCAGCTGA TGAGCGCCCT	1590
TTCCTTCTGG CACGTGACGG ACAAGATCCT ACCAGCTACC ACAGCAAACG CCTAAGAGCA	1650
GGAAAAATGT CTGCCAGGAA AGTGTCATT GGCCACATGG CCCCTGGCGC TCTGAGTCTT	1710
TGAGGAGTAA TCGCAAGCCT CGTTCAGCTG CAGCAGAAGG AAGGGCTTAG CCAGGGTGGG	1770
CGCTGGCGTC TGTGTTGAAG GGAAACCAAG CAGAAGCCAC TGTAATGATA TGTCACAATA	1830
AAACCCATGA ATGAAAAAAA AAAAAAAAAA AAAAAAAAAA AA	1872

FIGURE 1B

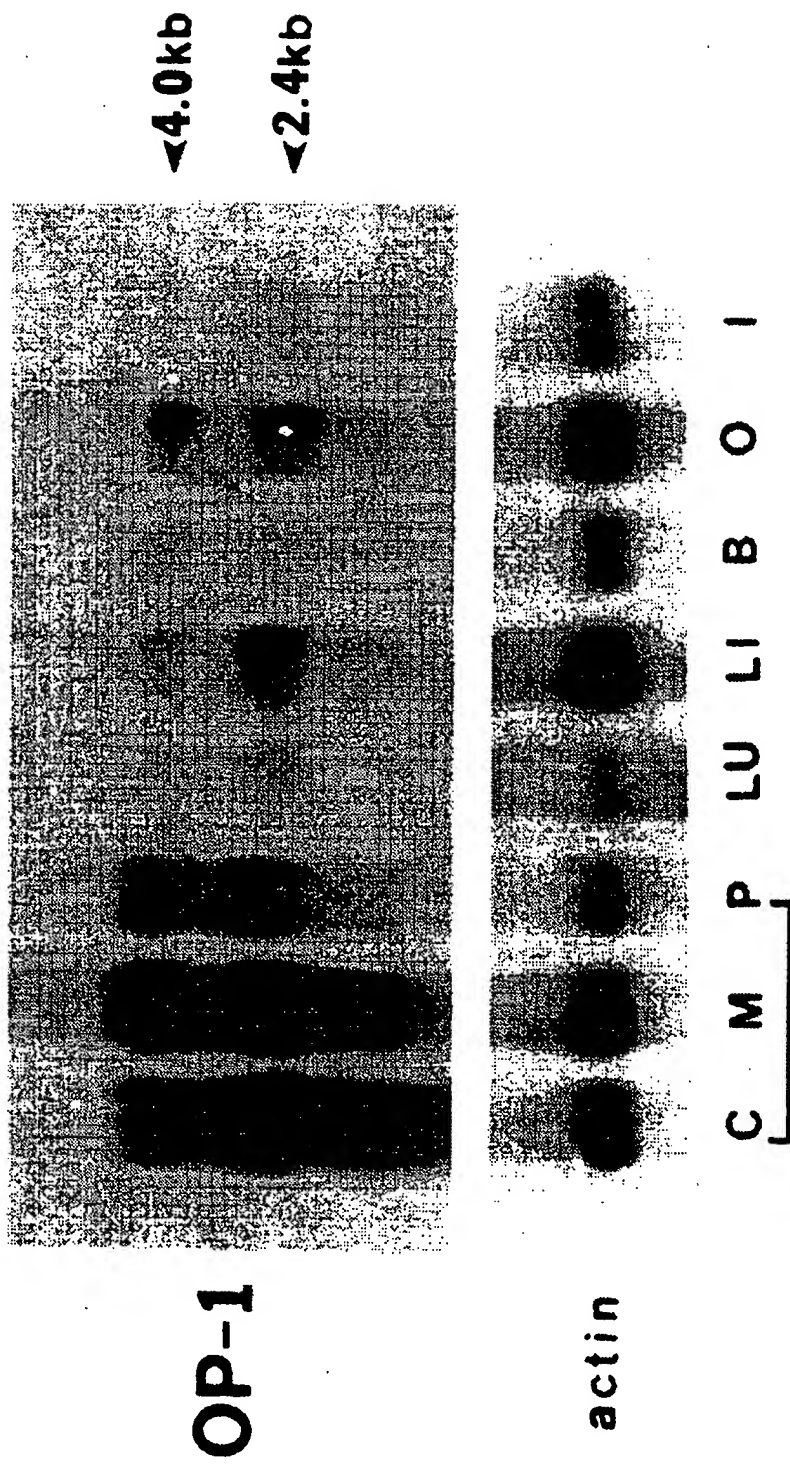


FIG. 2

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FIG. 3A

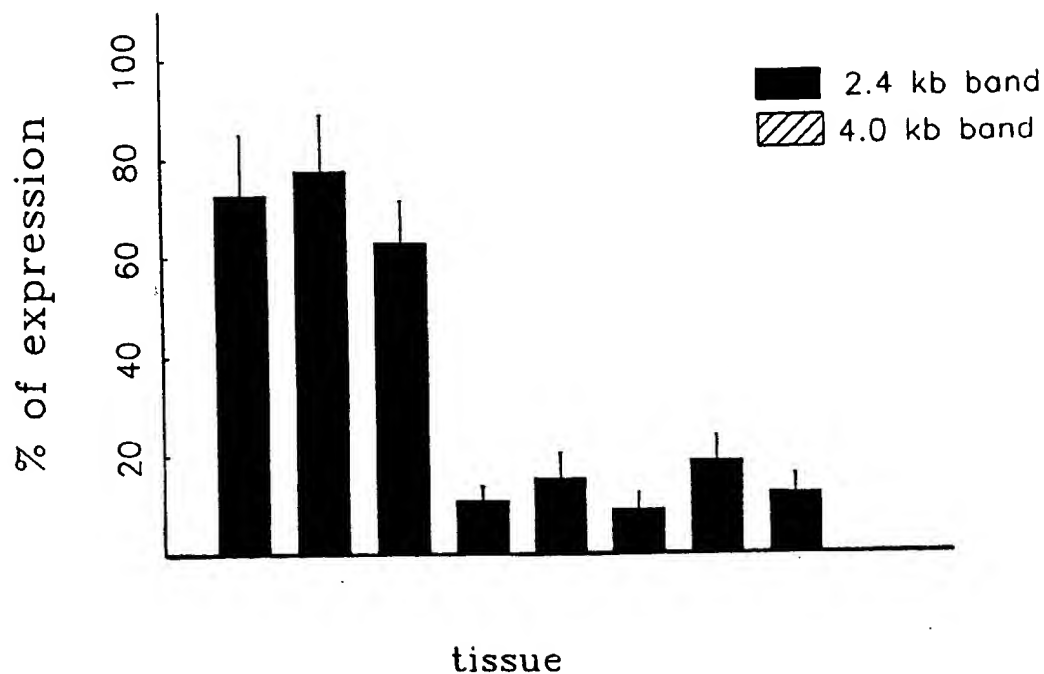
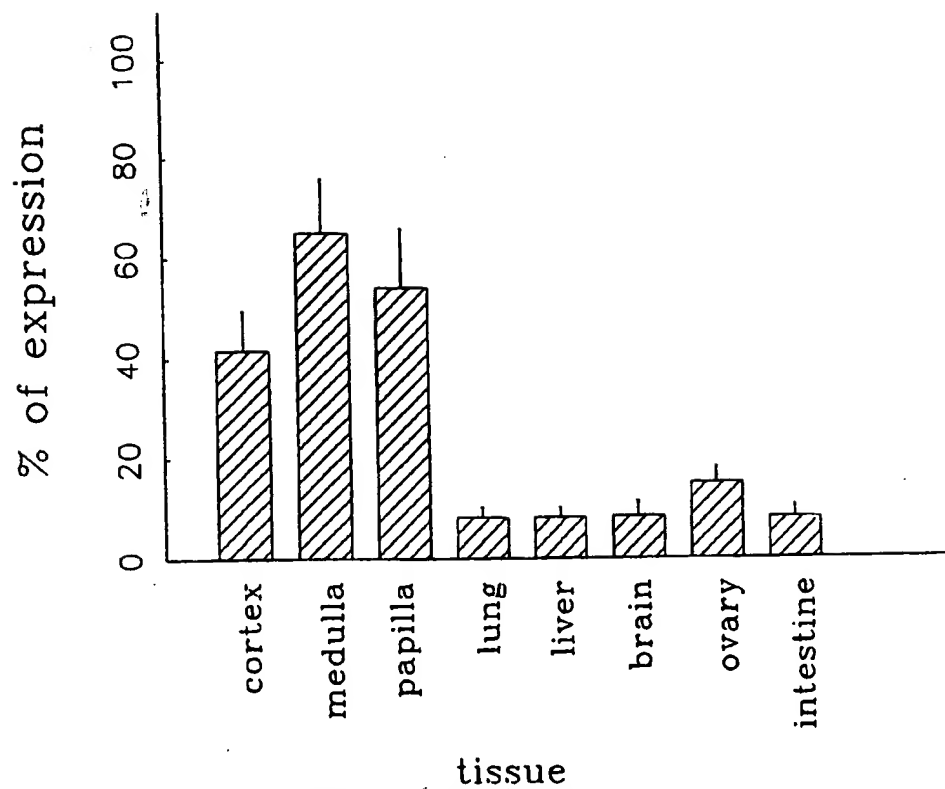


FIG. 3B



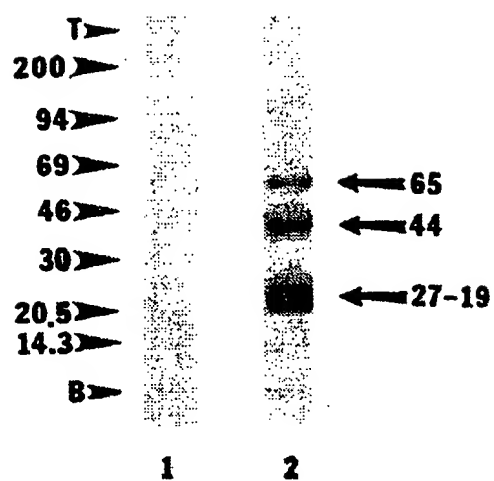


FIG. 4

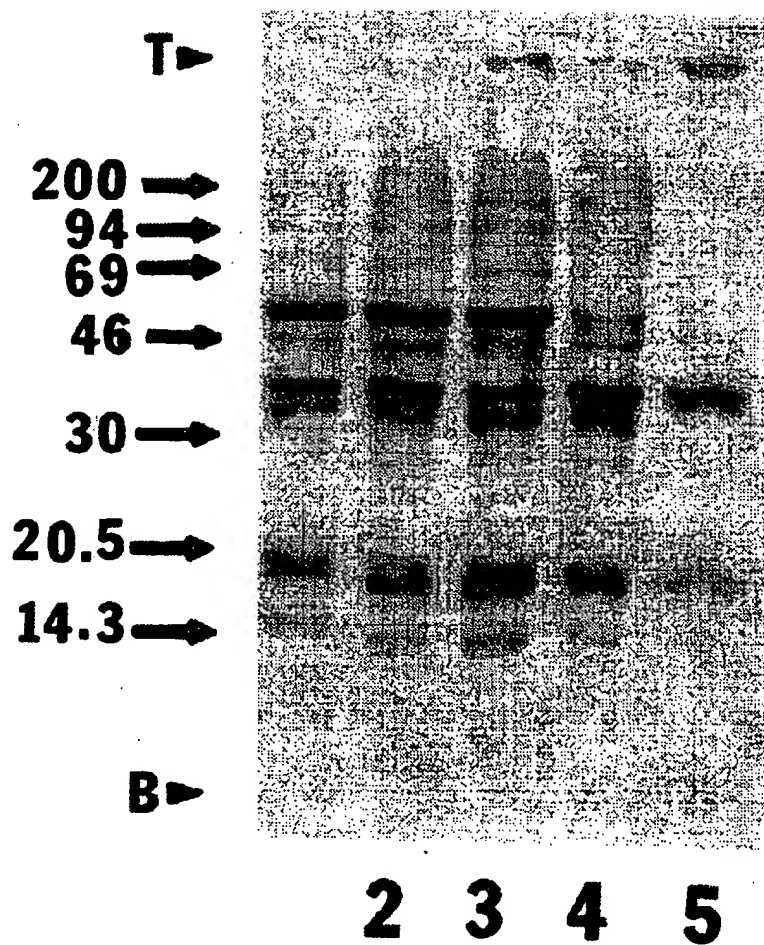


FIG. 5

FIG. 6B

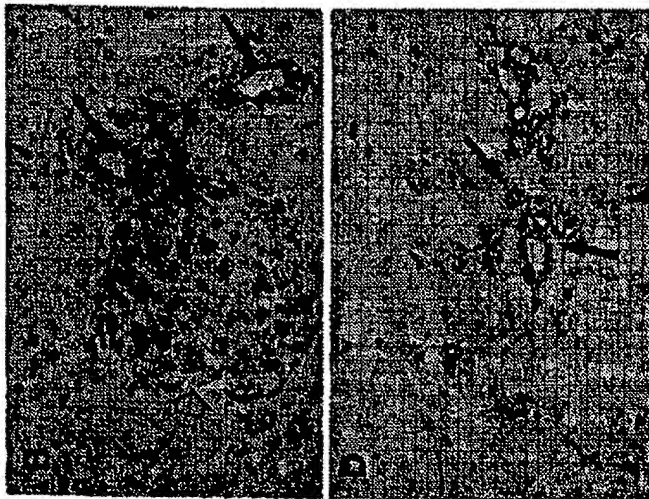


FIG. 6D

FIG. 6A

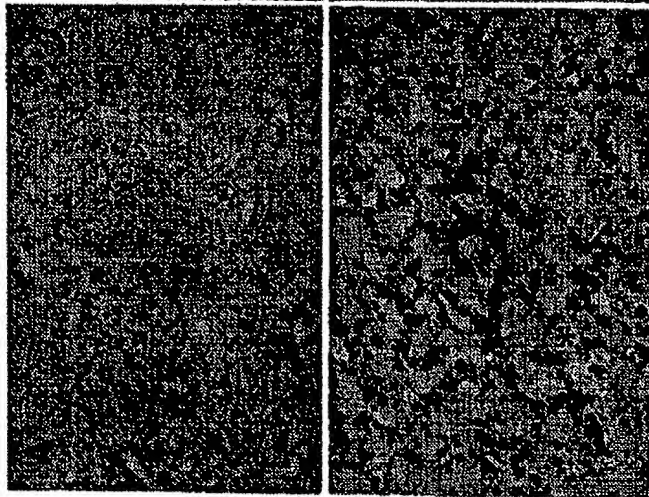
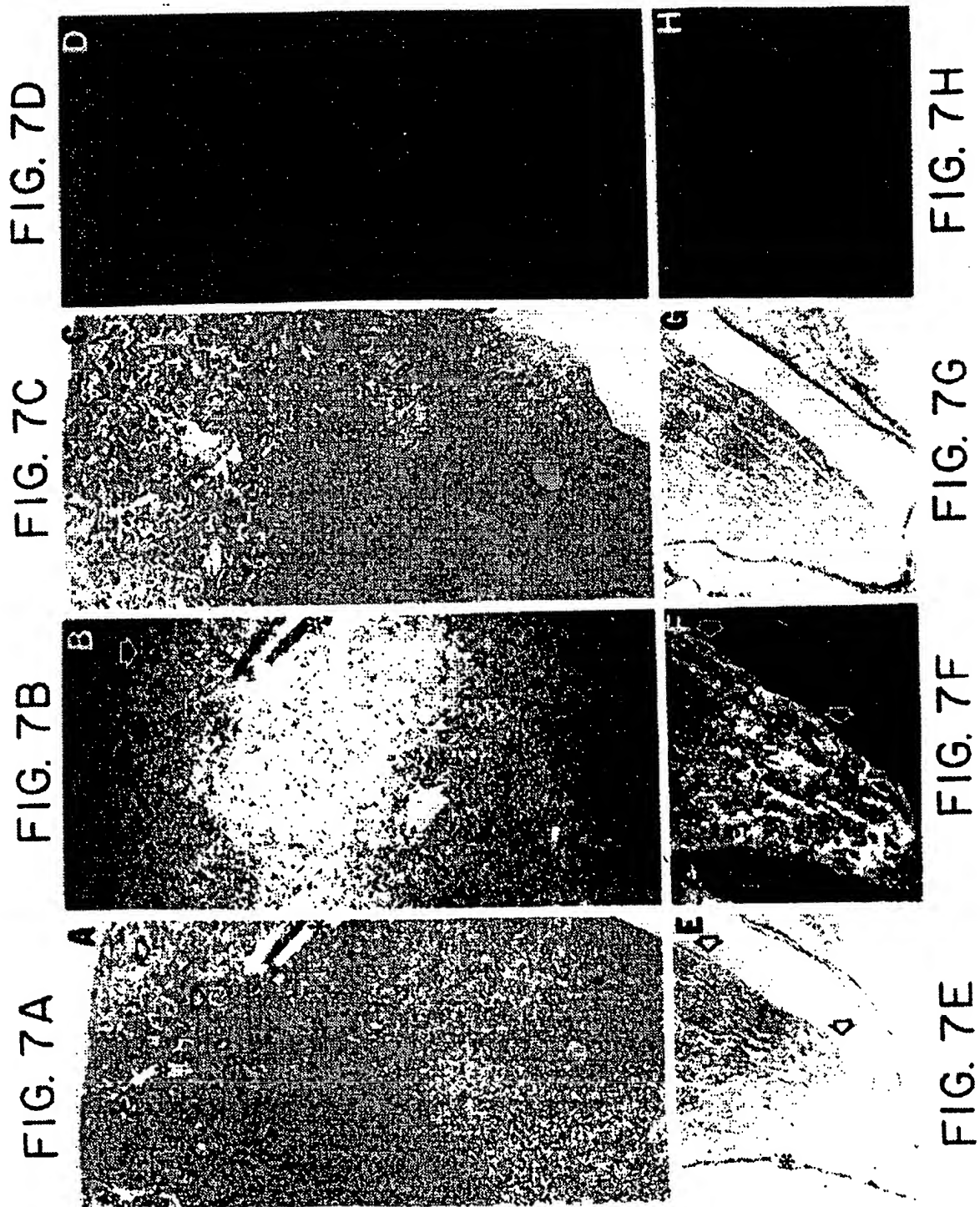


FIG. 6C



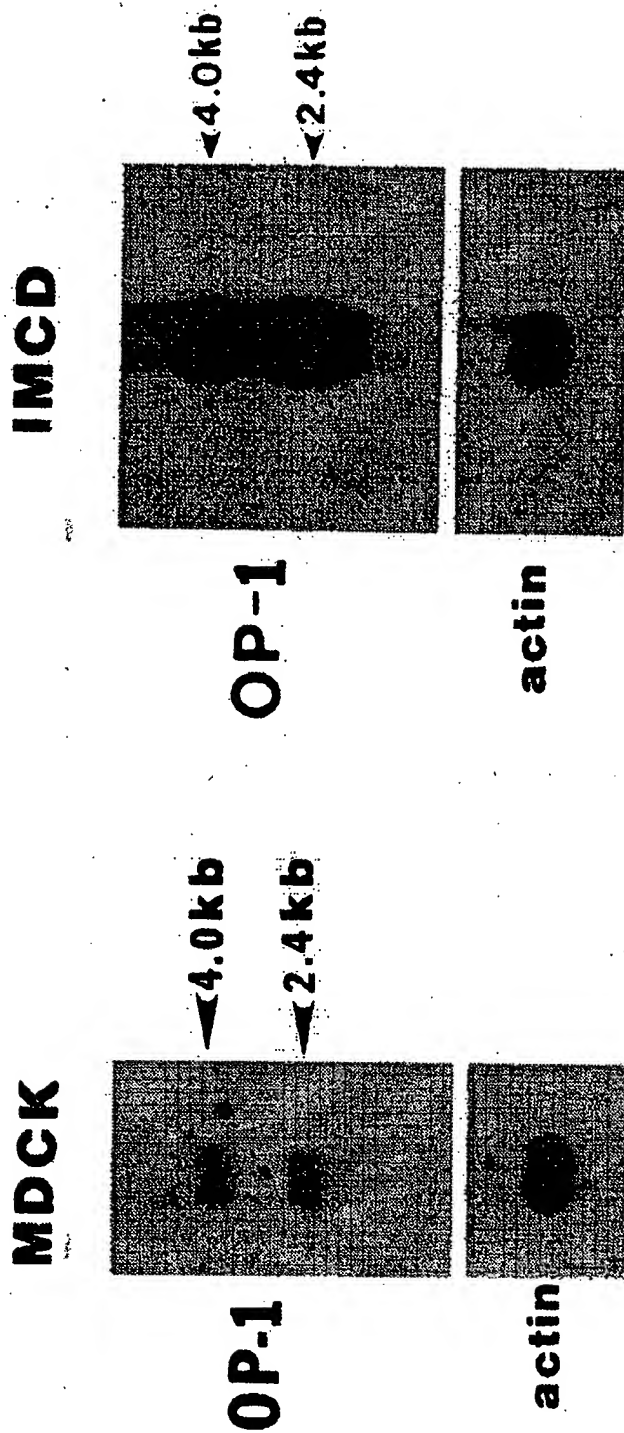


FIG. 8B

FIG. 8A

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/13215A. CLASSIFICATION F SUBJECT MATTER
IPC 6 C12Q1/02 C12Q1/68 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 05172 (CRATIVE BIOMOLECULES, INC.) 18 March 1993 the whole document ---	1-20
A	US,A,4 857 456 (URIST) 15 August 1989 the whole document --- -/--	21

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

7 March 1995

Date of mailing of the international search report

16. 03. 95

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Hornig, H

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/13215

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. BIOL. CHEM., vol.267, no.28, 5 October 1992, AM. SOC. MOL. BIOL., INC.,BALTIMORE, US; pages 20352 - 20362 T.K. SAMPATH ET AL. 'Recombinant human osteogenic protein (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro' cited in the application the whole document -----	1-21
P,X	WO,A,94 03600 (CREATIVE BIOMOLECULES, INC.) 17 February 1994 see claims 25-35 -----	21

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/ 13215

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ **Claims Nos.:**
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 17,18 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ **Claims Nos.:**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ **Claims Nos.:**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US 94/13215

Patent document cited in search report	Publication date	Patent family member(s)	Publication date	
WO-A-9305172	18-03-93	AU-A-	2564592	05-04-93
		AU-A-	2862492	05-04-93
		AU-A-	3176293	27-04-93
		CA-A-	2116559	01-04-93
		CA-A-	2116560	18-03-93
		CA-A-	2116562	18-03-93
		EP-A-	0601106	15-06-94
		EP-A-	0601129	15-06-94
		EP-A-	0601135	15-06-94
		JP-T-	6510989	08-12-94
		JP-T-	6510432	24-11-94
		WO-A-	9304692	18-03-93
		WO-A-	9305751	01-04-93

US-A-4857456	15-08-89	NONE		

WO-A-9403600	17-02-94	AU-B-	4795193	03-03-94
		AU-B-	4797193	03-03-94
		AU-B-	4995593	03-03-94
		AU-B-	5129293	12-04-94
		AU-B-	5129393	12-04-94
		AU-B-	5162393	12-04-94
		AU-B-	5290893	12-04-94
		AU-B-	5590094	24-05-94
		WO-A-	9403075	17-02-94
		WO-A-	9403200	17-02-94
		WO-A-	9406447	31-03-94
		WO-A-	9406399	31-03-94
		WO-A-	9406449	31-03-94
		WO-A-	9406420	31-03-94
		WO-A-	9410203	11-05-94
		AU-A-	3176293	27-04-93
		CA-A-	2116559	01-04-93
		EP-A-	0601135	15-06-94
		WO-A-	9305751	01-04-93
